Kidney International, Vol. 48 (1995), pp. 1344–1346

TECHNICAL NOTE

Thromboxane synthase expression co-localizes with infiltrating macrophages in renal allograft biopsies

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Thromboxane synthase (TS) catalyzes the formation of the eicosanoid thromboxane (TxA_2), which is a potent vasoconstrictor and activator of platelet aggregation. It has a widespread distribution, including hemopoietic cells (platelets, macrophages, monocytes) and many tissues, such as lung, liver, kidney, and spleen [1]. Cultured renal cells including mesangial cells, tubular epithelia, and medullary interstitial cells have also been found to produce TxA_2 [2]. TxA_2 has been proposed as a potential mediator of cyclosporine nephrotoxicity in renal allografts. In this light, the TS inhibitor, CGS 13080, has been used to attenuate the decrease in renal function caused by cyclosporine, but with mixed results [4, 5]. In human renal allografts undergoing acute or chronic rejection, we found thromboxane synthase expression in cells which appear to be infiltrating macrophages or monocytes by morphology [3]. This study describes an immunohistochemical technique utilized to confirm whether parenchymal renal cells or macrophages account for this TS enzyme expression. This method is based in histochemical double-labeling technique utilizing the immunoperoxidase avidin/biotin system and two monoclonal antibodies: Kon 7 and KP1. Kon 7 is a mouse monoclonal antibody directed against TS [2]. KP1 is a mouse anti-human monoclonal antibody directed against the CD68 antigen present in a lysosomal fraction of human lung macrophages and identifies macrophages and other phagocytic mononuclear cells [6].

Methods

Fourteen biopsy specimens from 14 renal allograft recipients who underwent a percutaneous renal allograft biopsy from January 1994 to June 1994 were used for this study. Indications for the biopsy include: rising serum creatinine or delayed graft function. Biopsies were obtained under ultrasonographic guidance using a modified Vim-Silverman needle or an automated biopsy gun. The tissues were fixed in a proprietary buffered formaldehyde ionized zinc solution (Z-fix, Anatech Ltd., Battle Creek, MI), embedded in paraffin wax, and processed for routine histological studies including hematoxylin-eosin and periodic acid-Schiff stains. The histopathologic diagnoses in this collection of 14 specimens include: acute rejection (5), chronic rejection (3), acute and chronic rejection (2), acute tubular necrosis (2), recurrent membranoproliferative glomerulonephritis type I (1), and recurrent diabetes (1).

In preparation for labeling with the first primary antibody, the tissue sections were first deparaffinated in xylene and washed with 100% ethanol, 95% ethanol, and 70% ethanol, followed by distilled water. Endogenous peroxidase activity was blocked with 0.3% H_2O_2 in methanol, followed by standard phosphate buffered saline (PBS) washes (10 mM Na PO_4, pH 7.5, 0.9% NaCl). The tissues were then blocked with 5% normal serum solution (NSS) from the Vectastain ABC/DAB Elite kit (mouse IgG type, Burlingame, CA). Excess serum was blotted, followed by a 30 minute incubation with the TS antibody, Kon 7 (courtesy of Dr. Volker Ullrich, Universitat Konstanz, Konstanz, Germany) at a dilution of 1:1000 in 5% NSS. The tissues were washed in PBS, and incubated with biotinylated secondary horse anti-mouse IgG antibody (Vectastain ABC/DAB Elite kit). After washing with PBS, ABC Reagent (Vectastain ABC/DAB Elite kit) followed by diaminobenzidine hydrotetrachloride (DAB) chromagen (brown color) were sequentially added, and finally washed with PBS.

In preparation for labeling these same tissue specimens with the secondary primary antibody, KP1, tissues were blocked with 5% NSS/PBS. KP1 was then applied at a 1:6 dilution in 5% NSS for 30 minutes, followed by a PBS wash. Secondary antibody (horse anti-mouse IgG) was again applied, followed by PBS wash, 5 minutes, 2 changes. ABC reagent was applied, followed by a PBS wash, and a second chromogen, Vector SG (blue color, Vector SG kit, Burlingame, CA) was applied, incubated, and rinsed with distilled water.

The tissue specimens were not counterstained with hematoxylin, since the counterstain can interfere with the chromogen color. They were mounted and coverslipped using Crystalmount medium.

Fourteen biopsies were double-labeled, while four of the fourteen were single-labeled with either Kon-7 or KP-1 and stained

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Received for publication November 14, 1994
and in revised form May 8, 1995
Accepted for publication May 8, 1995

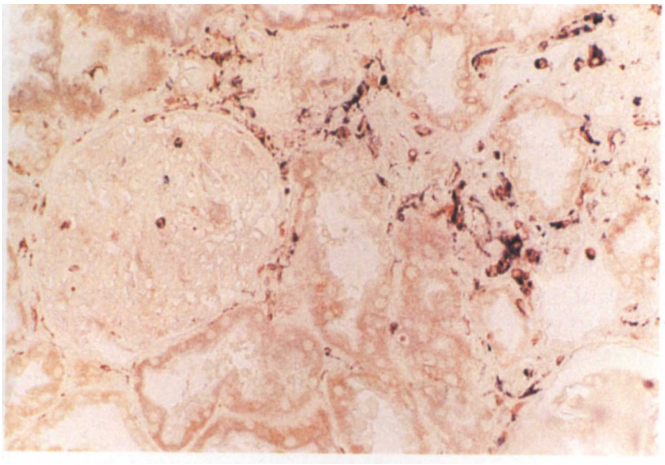

Fig. 1. *Low magnification view of cortex with glomerulus (left), tubules (center) and vascular plexus (arteriole and vein right center) showing inflammatory cell infiltrate.* The inflammation is present in glomeruli and around tubules and vessels [Stain Kon 7/DAB (brown) and KP1/Vector SG (blue); Magnification 210×].

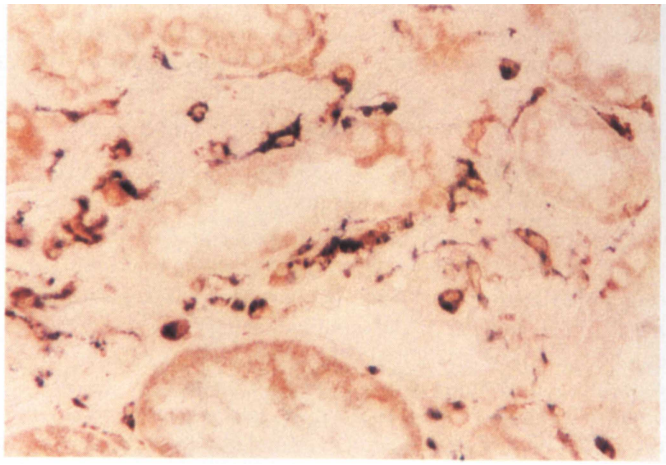

Fig. 2. *Intermediate magnification of tubules and vein with interstitial inflammation.* [Stain Kon 7/DAB (brown) and KP1/Vector SG (blue); Magnification 412×].

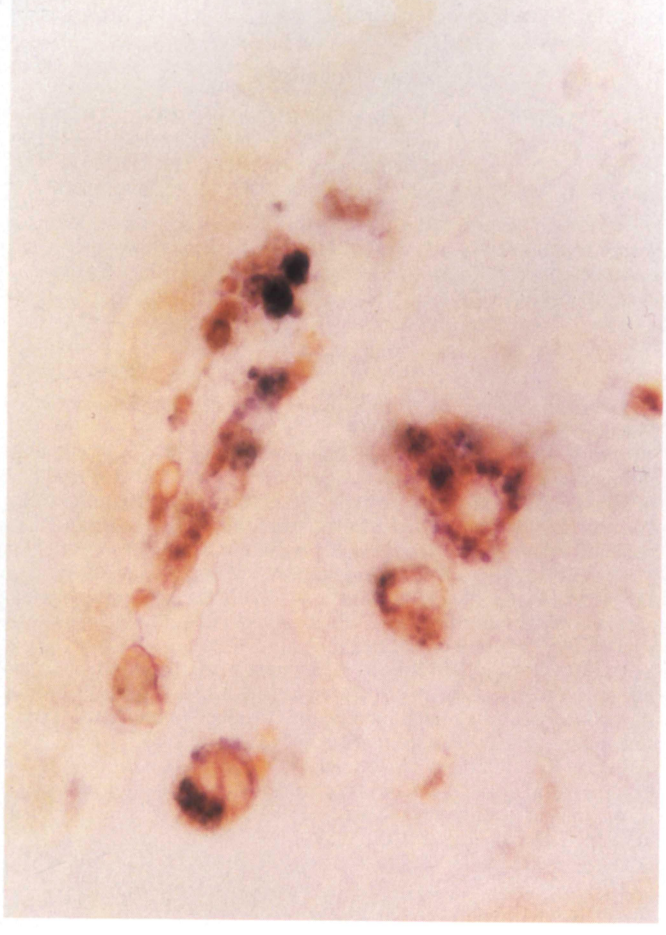

Fig. 3. *Biopsy specimen double-stained with Kon 7/DAB (brown) followed by KP1/Vector SG (blue).* Interstitial cells show staining with both brown and blue chromogens. (Magnification ×1020).

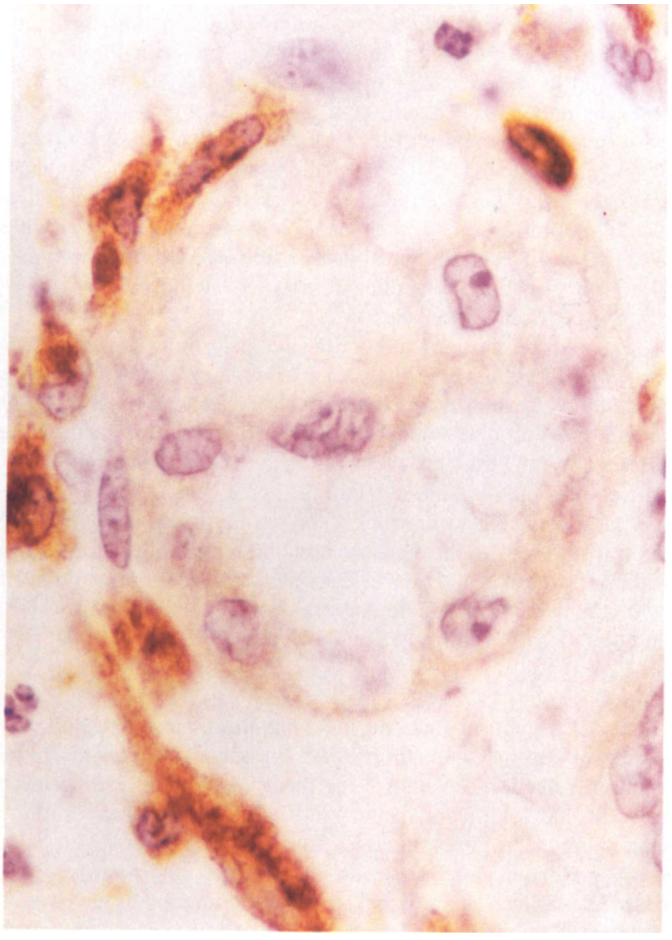

Fig. 4. *Biopsy specimen single-stained with Kon7/DAB (brown) and hematoxylin nuclear counterstain (blue).* Interstitial cells show staining with the brown chromogen. (Magnification ×1020).

with Vector SG (blue color) or DAB (brown color) to determine baseline staining and quality. Negative controls consisted of the above protocol without the application of either antibody. Normal renal tissue samples were also processed under the same conditions as the biopsy specimens as additional negative tissue controls.

Results

All of the biopsy specimens showed specific light blue or dark brown single- or double-antibody staining within the cytoplasm of cells found in the interstitium or glomerulus. The biopsy specimens which underwent the double-labeling procedure demonstrated numerous interstitial cells which stained dark brown for thromboxane synthase (Kon 7 positive, brown color) (Fig. 1). Under high magnification (Figs. 2, 3), cells appearing to stain for the TS antibody also showed blue granules, due to the staining of the macrophage marker (KP1, positive blue color). Therefore, all cells expressing thromboxane synthase (Kon 7, DAB chromogen, brown color) are macrophages based on detection of blue granules (KP1/Vector SG chromogen, blue color) in the cytoplasm. A single antibody control (Kon 7/DAB) is shown in Figure 4. The negative control showed very light brown staining found diffusely throughout the tissue section. The normal renal tissue showed no staining for either chromogen.

To exclude non-specific chromogen staining occurring by binding to unbiotinylated secondary antibody, the same specimens were processed omitting primary antibody but applying the secondary antibody and each chromogen separately. No staining was observed under these conditions.

To test for possible allosteric blocking of the KP1 site by the Kon 7 antibody, the same specimens were stained with each antibody alone and together, in reverse order (KP1 followed by Kon 7). Comparison of each condition revealed no noticeable variation in the quantity and type of cells stained. Furthermore, the pattern of double-labeling results was confirmed by single-labeling for each antibody.

To compare chromogen staining intensity between the DAB (brown) and Vector SG (blue), four of the fourteen biopsies were single-labeled using Kon 7 and DAB or Vector SG each. DAB stained more intensely compared to Vector SG, but each of the chromogens maintained their respective dark brown and light blue colors when double-labeling the same cell.

The labeled cells were found to be predominantly in patches of infiltrating cells in the cortical and medullary interstitium and within the glomeruli. Some specimens showed double-labeled cells completely surrounding several proximal and distal tubules, and occasionally within the tubular lumen.

Discussion

Our data confirm that the most significant and perhaps only source of thromboxane in episodes of acute and chronic renal allograft rejection are infiltrating macrophages, since only these cells labeled for thromboxane synthase. Thromboxane synthase expressing macrophages are also found in biopsy specimens with histologic diagnosis of acute tubular necrosis and recurrent disease. These macrophages are found predominantly in the cortical and medullary interstitium, but can also be found in the glomeruli and tubules. No consistent distinction in labeling pattern or quantity was found between biopsies with acute or chronic rejection, which suggests that thromboxane can play an important role in both conditions, potentially causing vasoconstriction by affecting the peritubular and glomerular capillary beds. Furthermore, thromboxane may be a potential cause of chronic nephropathy by decreasing renal perfusion and glomerular filtration rate, leading to irreversible chronic sclerosis. The experimental technique described here confirms that thromboxane synthase expressing cells and, hence, thromboxane synthase production in these conditions, is due to infiltrating macrophages. In addition, based on the absence of staining in the normal renal tissue specimens there appears to be no intrinsic thromboxane synthase expression in the normal kidney [3].

These double-labeling studies confirm the anatomic distribution of TS positive cells we previously reported in single-labeling studies [3]. In the previous study we used the Banff schema [7] to describe the distribution of TS positive cells and demonstrated that increased levels of infiltration predicted renal function impairment.

In summary, we have described a simple, reproducible method of identifying thromboxane synthase expression and specific localization of such expression using an immunohistochemical double-labeling procedure. Thromboxane synthase may play a broader role in renal diseases than previously expected. These studies may provide insight into the role of infiltrating macrophages in the pathogenesis of acute and chronic allograft nephropathy, as well as other conditions.

Acknowledgments

This study was supported in part by GCRC, MO1 RR00082. Dr. Byron P. Croker was supported in part by NIH Grant DK07518-06, and Dr. Christopher Wilcox was supported in part by NIH Grant DK36079. The authors thank Ms. Pam Schafer for revisions with the manuscript.

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